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13. ABSTRACT ***(Maximum 200 Words)***

The regulation of the Transforming Growth Factor-β (TGF-β) signaling pathway and its role in cancer is an area of intense research. We are investigating the regulatory role of casein kinase I (CKI) in the TGF-β signaling cascade. We have found that one family member inparticular, CKIε, binds to all Smads and the cytoplasmic domains of the Type I and Type II receptors both *in vitro* and *in vivo*. The interaction of CKIε with the Type I and Type II receptors is independent of TGF-β ligand stimulation. However, the CKIε/Smad interaction is transiently disrupted by TGF-β stimulation, with complete disassociation by 2 hours. Since CKIε is also a serine/threonine kinase, we examined *in vitro* phosphorylation of Smads and receptors by CKIε and found that only the receptor-activated Smads and the Type II Receptor are phosphorylated by CKIε. In addition, we have mapped the CKIε phosphorylation sites of Smad3 to the MH1 domain and the linker region. Furthermore, in the absence of TGF-β, transient overexpression of CKIε dramatically reduces basal transcriptional reporter activity, but in the presence of ligand CKIε increases TGF-β mediated transcription. Finally, CKIε is capable of significantly enhancing the transcriptional activity of smad3. Taken together, these observations provide exciting evidence for a functional role of CKIε in the TGF-β pathway, a pathway that has been shown to be involved in the development and progression of many different types of cancers.

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Table of Contents

Introduction

The Transforming Growth Factor-β (TGF-β) signaling pathway has been shown to be critical to the processes of embryological development of organisms as diverse as fruit flies and humans. This pathway can be detected at very early stages of development and acts to coordinate the complex mechanisms of cellular differentiation that will ultimately result in a mature organism. The TGF-β pathway continues its regulation of cellular events during the developmental stages and throughout the lifespan of more complex organisms. The power that this signaling cascade has over cellular fate is necessary for its ability to regulate development and differentiation, however when regulatory controls are lost, the result is usually uncontrolled growth and proliferation. Therefore, it is not surprising that mutations within the TGF-β pathway have been implicated in a wide range of clinically observed oncogenic lesions including breast cancer.

The TGF-β superfamily of ligands includes the bone morphogenetic proteins (BMPs), activin and TGF-β. The signaling pathway is a relatively simple cascade that consists of the ligand, the type I and type II receptors, and the cytoplasmic signal transducers called smads (for a more detailed review of this pathway refer to references 54-56) . The type I and type II receptors are serine/threonine kinases that, upon ligand binding, form a heterotetrameric complex in which the constitutively active type II receptor phosphorylates the type I receptor in the GS domain resulting in catalytic activation. The activated type I receptor then transiently associates with and phosphorylates the receptor activated smads (R-smads) on their two most C-terminal serine residues. The smad proteins consist of two highly conserved mad homology domains, termed MH1 and MH2, connected by a relatively divergent linker region. The MH1 domain is involved in DNA binding, while the MH2 domain is important for protein/protein interactions. The mad homology domains are capable of interacting with each other in an inhibitory fashion that is alleviated by type I receptor phosphorylation. This phosphorylation results in association with the co-smad, translocation to the nucleus, and regulation of gene transcription usually through association with

coactivators, corepressors, or other transcription factors such as AP-1 or the Wnt regulated Lef/Tcf family members.

The casein kinase I (CKI) family has seven identified isoforms (α, β, γ1, γ2, γ3, δ, and ε) that possess a highly homologous N-terminal kinase domain and a highly divergent C-terminal tail, and have a predicted molecular weight of approximately 40-50kDa (for a detailed view of the CKI family refer to reference 1). These kinases have been implicated in a wide range of cellular functions including, vesicular trafficking, DNA damage repair, cell cycle progression, and cytokinesis. CKI was one of the first serine/threonine kinases ever purified and hence extensive research has been done on characterizing its activity, substrate specificity, function, tissue distribution, subcellular localization and regulation. The results of this research have lead to the characterization of the general consensus phosphorylation sequence S/T/Y(P)X_{1-3}S/T (40,41). This sequence suggests that the action of other kinases is probably required for CKI activity, and thus CKI has been classified as a phosphate-directed kinase. Since it is not clear how these constitutively active kinases are regulated within a cell, it appears that this may be one major mechanism by which control is achieved, with subcellular localization being a second likely mechanism. The CKI family also possesses two other potentially interesting physical features, the first being a kinesin homology domain (KHD) and the second being a near consensus SV40 T antigen nuclear localization sequence (NLS) (1). The significance of these sequences remains to be determined however, a recent paper has demonstrated that the NLS sequence is definitely functional and necessary for nuclear translocation of CKIα (27). The majority of the research done on the CKI family has focused primarily on characterizing their function and identifying potential substrates. However, over the last several years there has been a revolution in the CKI field and this obscure family of kinases has moved into a position of intense research in the field of signal transduction. The last several years have seen the publication of numerous papers that demonstrate a significant role for CKIε and CKIδ in the circadian rhythms of mammals (17-19), the cytoplasmic sequestration of NFAT and the regulation of a $G_{q/11}$-coupled

receptor by CKIα (25,26), the regulation of the β-PDGFR by CKIγ2 (23), and the positive regulation of the Wnt signaling pathway by CKIε (9-12). These findings combined with our own preliminary results has resulted in our undertaking the task of determining if the CKI family plays a functional role in the TGF-β signaling pathway and what the significance of this role may mean with respect to the development and progression of breast cancer.

Body/Results

Task 1:

Determine if CKI family members can physically interact with components of the TGFβ pathway and whether these interactions are ligand dependent.

Several years ago a yeast two-hybrid screen was performed in the lab using smad3 as bait. This screen generated several hundred clones, each representing a potential smad3 interactor and TGF-β pathway effector. The results of this screen have since acted as a launching pad for further investigation into identifying these genes and elucidating their potential role within the TGF-β pathway. One gene that has been identified on at least three separate occasions is Casein Kinase Iγ2 (CKIg2). The identification of this kinase as a potential interactor was interesting in and of itself, but with the publication of a study implicating CKIε (a closely related homolog of CKIγ2) in the positive regulation of the Wnt pathway (9-12), our interest increased dramatically. We were curious to see if CKIε could also interact with smads and play a role in regulating the TGF-β pathway.

Full length CKIε interacts strongly with Smads *in vitro,* while full length CKIα/δ/γ2 family members interact weakly.

CKI family members were radiolabled *in vitro* with Methionine-S^{35} and GST pulldown assays were performed using purifed GST fusions of the receptor activated smad proteins. As shown in Figure 1, CKIε is able to bind strongly to smad1/2/3, but weakly to smad5. Furthermore, using GST constructs that are fused to either the MH2 domain (S3C) or the MH1 domain and linker region (S3NL) of smad3, it appears that CKIε has a much higher affinity for the MH2 domain (Fig. 1). This point may prove important as a functional role for CKIε in the TGF-β pathway is developed. In addition, I have looked for *in vitro* interaction with smad4, the co-smad, and found

that CKIε also can interact strongly with this smad (data not shown). In addition, I have repeated these GST pulldown assays for each CKI family member that has been shown to be expressed in the TGF-β responsive cell lines that we use. I have found that full length CKIα/δ family members bind weakly to GST purified smads in comparison to CKIε binding, while the full length CKIγ2 family member had almost undetectable binding. This last finding was especially surprising, since this was the isoform that was originally identified in the yeast two-hybrid screen in which smad3 was used as bait. Further analysis of the CKIγ2 sequences that were isolated in the yeast two-hybrid assay showed that none of them contained the N-terminal portion of the protein. Therefore, I fused CKIγ2 lacking the N-terminus to GST and performed a GST pulldown assay using smads radiolabled with Methionine-S^{35}. As shown in Figure 2, the elimination of the N-terminus of CKIγ2 resulted in strong binding to smad1, detectable binding to smads3/4, but no detectable binding to smad2. This data suggests that the unique extension preceding the kinase domain that is found at the N-terminus of CKIγ2 might somehow inhibit *in vitro* binding with smads.

Full length CKIε can interact with the TGF-β Type I and Type II Receptors *in vitro*.

It was previously observed that immunoprecipitated TGF-B type II receptors possessed, what was believed at the time to be, an intrinsic casein kinase I activity (39). Close examination of the data shows a coimmunoprecipitated protein of about 40-45kDa, the approximate size of CKIε. This observation lead me to hypothesize that CKIε may be capable of interacting with the TGF-B type I and type II receptors. Therefore, CKIε was again radiolabled *in vitro* with Methionine-S^{35} and GST pulldown assays were performed with purifed GST fusions of the cytoplasmic domains

of the TGF-β type I and type II receptors, as well as, a GST fusion of a BMP type I receptor. As shown in Figure 3, CKIε is capable of binding to both type I and type II receptors.

CKIε can interact with Smads and TGF-β Type I and Type II Receptors *in vivo.*

Since CKIε can bind to smads and receptors *in vitro,* we next decided to look whether this interaction also occurred in living cells. HaCaT-CKIe3 cells, a spontaneously immortalized human keratinocyte cell line that is responsive to TGF-β ligand and stably transfected with CKIε, were used for these co-immunoprecipitation assays. Cell lysates were incubated with one of the following; anti-TGF-β type I, anti-TGF-β type II, anti-smad 2/3 or anti-smad 1/5, and then blotted for CKIε. As shown in Figure 4, CKIε can interact with receptors and smads *in vivo.* Furthermore, it appears that the strongest interaction occurs with the receptors, while a weaker interaction is seen with the receptor activated smads. We have also done a co-immunoprecipitation assay with anti-smad4 and found that CKIε and smad4, the co-smad, can also interact *in vivo.* Although parental HaCaT cells express CKIε endogenously and association with smads and receptors can be observed when co-IP experiments are done using the wild type cells, we decided to use the HaCaT-CKIe3 cells for these studies so that the interactions would be easier to detect and monitor. This point becomes most important when the co-IP experiment is done with the smad antibodies, due to the relatively weak signal observed with endogenous proteins alone (Figure 3).

TGF-β treatment transiently disrupts the CKIε/Smad interaction, but does not effect the CKIε/Receptor interaction.

The *in vivo* interaction with components of the TGF-β pathway provides some evidence that there may be a functional role played by CKIε. The next question we wanted to address was

whether treatment of the stably transfected HaCaT cells with TGF-β ligand might affect these interactions. Cells were treated over a four hour period with TGF-β ligand, the cells were lysed, lysates were incubated with anti-smad2/3, and blots were performed for CKIε. As shown in Figure 5, the interaction between CKIε and Smad2/3 is transiently disrupted with TGF-β treatment. However when the same experiment was performed for the TGF-β type II receptor, there was no observable disruption of CKIε binding (Figure 6). I have also performed this experiment probing for the TGF-β type I receptor and again found that there is no observable disruption of CKIε binding (data not shown).

Task 2:

Examine whether CKI family members can regulate TGF-β mediated gene transcription.

In order to determine if CKIε was capable of playing a functional role in the TGF-β pathway, we decided to use the classical transcription reporter assay as a measure of function. There are several reporter constructs that are widely used to monitor TGF-β regulated transcription. We tried two different constructs, the first is the 3TP-Lux construct that consists of a region of the PAI-1 promoter that is known to contain smad binding elements as well as AP-1 binding elements (47). The second is a cancatemerized smad binding element (SBE) fused to the luciferase gene, with no AP-1 sites present. HepG2 cells, a human hepatocellular carcinoma cell line, are responsive to TGF-β and easily transfectable. Using the SBE reporter construct, I found that adding just CKIε alone resulted in reduced basal transcriptional activity, and when TGF-β was added the transcriptional activity was actually enhanced. This resulted in a dramatic increase in fold TGF-β induction with the addition of CKIε compared to control. Furthermore, a kinase

dead construct of CKIε (CKIε-KD) was also capable of reducing basal transcriptional activity, but failed to enhance activity in response to TGF-β (Figure 7 and data not shown). This data suggests that the reduction in basal activity only requires the presence of the protein and not the kinase activity, while enhancement of TGF-β ligand treatment requires the protein and the kinase activity. In addition, using the 3TP-Lux reporter, HepG2 cells show a significant increase in transcriptional activity in response to TGF-β, however when smad3 and CKIε are added together the response to TGF-β is increased by approximately twice that seen with TGF-β alone (Figure 8). Furthermore, when Smad2 and CKIε were added together there was no enhancement over that seen for smad2 alone (Figure 8). The enhancement of smad3 transcriptional activity was also observed using the SBE-Lux reporter (Figure 9). These results imply that the DNA binding ability of smad3 is important for the ability of CKIε to enhance its transcriptional activity.

Task 3:

Determine if CKI family members are necessary to maintain normal TGF-β pathway function.

There are no reportable research accomplishments for this task for the time period addressed in this report.

Task 4:

Determine if CKI family members can phosphorylate components of the TGF-β pathway, identify potential phosphorylation sites, and evaluate the functional significance of these sites.

Since CKIε is a serine/threonine kinase (same as the TGF-β type I and type II receptors), we wanted to see if CKIε could phosphorylate purified smads *in vitro*. We were also curious to see if CKIε might be able to phosphorylate either of the receptors, since we have seen that they can

interact *in vitro* and *in vivo*. As shown in Figure 10, CKIε phosphorylates the TGF-β activated smads (smads2/3) and the BMP activated smads (smads1/5) to a lesser extent, but it does not phosphorylate the co-smad (smad4). In addition, we observed that CKIε appears to phosphorylate the MH1 domain and the linker region of smad3, but not the MH2 domain (the region phosphorylated by the type II receptor). Furthermore, CKIε can phosphorylate the cytoplasmic region of the type II receptor, but does not appear to phosphorylate the type I receptor (this observation remains to be resolved because purification of a reasonable amount of the kinase dead TGF-β type I receptor has proven challenging).

Key Research Accomplishments

- Shown that Casein Kinase Iε (CKIε) is capable of interacting with multiple components of the TGF-β signaling pathway both *in vitro* and *in vivo*.
- Shown that CKIε binding to the receptor activated Smads *in vivo* is transiently disrupted by TGF-β ligand stimulation.
- Shown that CKIε binding to the TGF-β type I and type II receptors is independent of TGF-β ligand stimulation.
- Shown that CKIε acts to regulate TGF-β mediated transcription, as well as enhance the transcriptional activity Smad3.
- Shown that CKIε can phosphorylate the receptor activated smads and the cytoplasmic domain the TGF-β type II receptor *in vitro*.
- Mapped the CKIε phosphoryaltion sites of Smad3 to the MH1 domain and the linker region.

Reportable Outcomes

Abstracts:

Casein Kinase Iε Regulates the TGF-β Pathway and Provides a Link for TGF-β Activation of the Wnt Pathway. David S. Waddell, Nicole T. Liberati, Jeremy N. Rich, and Xiao-Fan Wang. *Duke University Medical Center, Department of Pharmacology and Cancer Biology, Durham, NC 27710.* Submitted for the April 2002 AACR Meeting.

Casein Kinase Iε Plays a Functional Role in the Transforming Growth Factor-β Signaling Pathway. David S. Waddell, Nicole T. Liberati, Ph.D., and Xiao-Fan Wang, Ph.D. *Duke University Medical Center, Department of Pharmacology and Cancer Biology, Durham, NC 27710.* Submitted for the September 2002 Era of Hope Meeting.

Degrees:

Mrs. Xuefang Bai, the original recipient of this award, was funded for one year prior to graduating with a Master of Science Degree in Molecular Cancer Biology.

Cell Lines and Serum:

HaCat cells, a spontaneously immortalized cell line that is responsive to TGF-β, has been stabling transfected with CKIε and CKIγ2, as well as the kinase dead versions of these two proteins.

A polyclonal antibody has been raised in rabbits to the C-terminus of CKIγ2.

Conclusions

The TGF-β signaling pathway has been shown to be involved in a wide range of biological processes, including development, differentiation and oncogenesis. The regulation of this pathway, and its role in cancer, continues to be an area of intense investigation. Recently Casein Kinase Iε (CKIε) has been shown to positively control the Wnt pathway, another major pathway involved in the development of numerous types of cancers. In this study, we are engaged in an ongoing investigated to determine the regulatory role of CKIε in the TGF-β pathway. This pathway consists of the ligand, the Type I and the Type II serine/threonine receptor kinases, which complex upon ligand binding, to activate a family of intracellular signal transducing proteins called Smads. We have found that CKIε binds to all Smads and the cytoplasmic domains of the Type I and Type II receptors both *in vitro* and *in vivo*. The interaction of CKIε with the Type I and Type II receptors is independent of TGF-β ligand stimulation. However, the CKIε/Smad interaction is transiently disrupted by TGF-β stimulation, with complete disassociation by 2 hours. Since CKIε is also a serine/threonine kinase, we examined *in vitro* phosphorylation of Smads and receptors by CKIε. Only the receptor-activated Smads (Smads 1, 2, 3, and 5) and the Type II Receptor are phosphorylated by CKIε. Furthermore, in the absence of TGF-β, CKIε dramatically reduces basal transcriptional reporter activity, but in the presence of ligand CKIε increases TGF-β mediated transcription. Finally, the enhancement of TGF-β mediated transcription is most likely the result of the ability of CKIε to dramatically enhance Smad3 transcriptional activity. The potential mechanism by which the basal transcriptional activity is reduced remains unknown, but it is a major aim that we hope to determine with this

research. The fact that CKIε appears to play a role in controlling the basal activity of the TGF-β pathway, implies that this family of proteins may act as an important negative regulator of this pathway in the absence of ligand. The importance of this observation remains to be determined, however if CKIε does prove to be a necessary negative regulator, then this would be the first evidence that the CKI family may play some role in the development and/or progression of cancers in which there is a loss of regulation of the TGF-β pathway. These results taken together demonstrate that CKIε interacts with several components of the TGF-β pathway and plays a significant regulatory role in the presence and absence of ligand. These observations provide intriguing insight into the regulation of a major signal transduction pathway involved in the development and progression of many different types of cancers, including breast cancer.

Importance and Implications

The ongoing advances in the understanding and treatment of cancer depend almost unconditionally on the knowledge gained through basic scientific research conducted everyday by countless labs around the world. The increased understanding of how signal transduction pathways work and how mutations in these pathways can ultimately result in uncontrolled cell growth is invaluable to our ability to identify targets for the development of new drugs and improved treatments. The research described above is merely another cog in the wheel of our understanding of the TGF-β signaling pathway. Taken independently this research may seem trivial and insignificant, but when combined with the vast knowledge that we have already accumulated it becomes much more important as we try to determine how this signaling pathway functions and where mutations within this pathway may prove to be the most damaging with

respect to regulation. Whether the casein kinase I family ultimately proves to be a major player in the regulation of the TGF-β pathway, or just another minor effector remains to be determined. Regardless of the importance of the casein kinase I family within the framework of the TGF-β signaling pathway, it is becoming more and more apparent that this family of serine/threonine kinases has some significant role to play in the maintenance and regulation of many important and potentially oncogenic signal transduction pathways. This observation alone, irrespective of the role CKI may have in the TGF-β pathway, may someday make the CKI family members an important target in treating patients with cancer.

References

1. Gross, S.D. and Anderson, R.A. Casein Kinase I: Spatial Organization and Positioning of a Multifunctional Protein Kinase Family. *Cell. Signal.* **10,** 699-711 (1998).

2. Furuhashi, M., Yagi, K., Yamamoto, H., Furukawa, Y., Shimada, S., Nakamura, Y., Kikuchi, A., Miyazono, K., and Kato, M. Axin Facilitates Smad3 Activation in the Transforming Growth Factor β Signaling Pathway. *Mol. Cell. Biol.* **21,** 5132-5141 (2001).

3. Gietzen, K.F. and Virshup, D.M. Indentification of Inhibitory Autophosphorylation Sites in Casein Kinase Iε. *J. Biol. Chem.* **274,** 32063-32070 (1999).

4. Rubinfeld, B., Tice, D.A., and Polakis, P. Axin-dependent Phophorylation of the Adenomatous Polyposis Coli Protein Mediated by Casein Kinase Iε. *J. Biol. Chem.* **276,** 39037-39045 (2001).

5. Fish, K.J., Cegielska, A., Getman, M.E., Landes, G.M., and Virshup, D.M. Isolation and Characterization of Human Casein Kinase 1ε (CKI), a Novel Member of the CKI Gene Family. *J. Biol. Chem.* **270,** 14875-14883 (1995).

6. Cegielska, A., Geitzen, K.F., Rivers, A., and Virshup, D.M. Autoinhibition of Casein Kinase Iε (CKIε) Is Relieved by Protein Phosphatases and Limited Proteolysis. *J. Biol. Chem.* **273,** 1357-1364 (1998).

7. Graves, P.R. and Roach, P.J. Role of COOH-terminal Phosphorylation in the Regulation of Casein Kinase Iδ. *J. Biol. Chem.* **270,** 21689-21694 (1995).

8. Rivers, A., Gietzen, K.F., Vielhaber, E., and Virshup, D.M. Regulation of Casein Kinase Iε and Casein Kinase Iδ by an *in Vivo* Futile Phosphorylation Cycle. *J. Biol. Chem.* **273,** 15980-15984 (1998).

9. Kishida, M., Hino, S., Michiue, T., Yamamoto, H., Kishida, S., Fukui, A., Asashima, M., and Kikuchi, A. Synergistic Activation of the Wnt Signaling Pathway by Dvl and Casein Kinase Iε. *J. Biol. Chem.* **276,** 33147-33155 (2001).

10. Lee, E., Salic, A., and Kirschner. M.W. Physiological regulation of β-catenin stability by Tcf3 and CKIε. *J. Cell Biol.* **154,** 983-993 (2001).

11. Sakanaka, C., Leong, P., Xu, L., Harrison, S.D., and Williams, L.T. Casein Kinase Iε in the Wnt pathway: Regulation of β-catenin function. *Proc. Nat. Acad. Sci.* **96,** 12548-12552 (1999).

12. Peters, J.M., McCay, R.M., McCay, J.P., and Graff, J.M. Casein Kinase I transduces Wnt signals. *Nature* **401,** 345-349 (1999).

13. Li, L., Yuan, H., Xie, W., Mao, J., Caruso, A.M., McMahon, A., Sussman, D.J., and Wu, D. Dishevelled Proteins Lead to Two Signaling Pathways. *J. Biol. Chem.* **274,** 129-134 (1999).

14. Zhang, Y., Qiu, W.J., Liu, D.X., Neo, S.Y., He, X., and Lin, S.C. Differential Molecular Assemblies Underlie the Dual Function of Axin in Modulating the Wnt and JNK Pathways. *J. Biol. Chem.* **276,** 32152-32159 (2001).

15. Zhang, Y., Neo, S.Y., Han, J., and Lin, S.C. Dimerization Choices Control the Ability of Axin and Dishevelled to Activate c-Jun N-terminal Kinase/Stress-activated Protein Kinase. *J. Biol. Chem.* **275,** 25008-25014 (2000).

16. Engel, M.E., McDonnell, M.A., Law, M.A., and Moses, H.L. Interdependent SMAD and JNK Signaling in Transforming Growth Factor-β-mediated Transcription. *J. Biol. Chem.* **274,** 37413-37420 (1999).

17. Vielhaber, E., Eide, E., Rivers, A., Gao, Z.H., and Virshup, D.M. Nuclear Entry of the Circadian Regulator mPER1 Is Controlled by Mammalian Casein Kinase Iε. *Mol. Cell. Biol.* **20,** 4888-4899 (2000).

18. Keesler, G.A., Camacho, F., Guo, Y., Virshup, D., Mondadori, C., and Yao, Z. Phosphorylation and destabilization of human period I clock protein by human casein kinase Iε. *NeuroReport.* **11,** 951-955 (2000).

19. Lowrey, P.L., Shimomura, K., Antoch, M.P., Yamazaki, S., Zemenides, P.D., Ralph, M.R., Menaker, M., and Takahashi, J.S. Positional Syntenic Cloning and Functional Characterizing of the Mammalian Circadian Mutation tau. *Science* **288,** 483-491 (2000).

20. Zilian, O., Frei, E., Burke, R., Brentrup, D., Gutjahr, T., Bryant. P.J., and Noll, M. *double-time* is identical to *discs overgrown*, which is required for cell survival, proliferation and growth arrest in *Drosophila* imaginal discs. *Development.* **126,** 5409-5420 (1999).

21. Kitabayashi, A.N., Kusuda, J., Hirai, M., and Hashimoto, K. Cloning and Chromosomal Mapping of Human Casein Kinase I γ2 (CSKN1G2). *Genomics.* **46,** 133-137 (1997).

22. Lussier, G. and Larose, L. A Casein Kinase I Activity is Constitutively Associated with Nck. *J. Biol. Chem.* **272,** 2688-2694 (1997).

23. Bioukar, E.B., Marricco, N.C., Zuo, D., and Larose, L. Serine Phosphorylation of the Ligand-activated β-Platelet-derived Growth Factor Receptor by Casein Kinase I-γ2 Inhibits the Receptor's Autophosphorylating Activity. *J. Biol. Chem.* **274,** 21457-21463 (1999).

24. Voisin, L., Larose, L., and Meloche, S. Angiotension II stimulates serine phophorylation of the adapter protein Nck: physical association with serice/threonine kinases Pak1 and casein kinase I. *Biochem. J.* **341,** 217-233 (1999).

25. Zhu, J., Shibasaki, F., Price, R., Guillemot, J.C., Yano, T., Dotsch, V., Wagner, G., Ferrara, P., and McKeon, F. Intramolecular Masking of Nuclear Import Signal on NF-AT4 by Casein Kinase I and MEKK1. *Cell.* **93,** 851-861 (1998).

26. Budd, D.C., McDonald, J.E., and Tobin, A.B. Phosphorylation and Regulation of a $G_{q/11}$-coupled Receptor by Casein Kinase Iα. *J. Biol. Chem.* **275,** 19667-19675 (2000).

27. Fu, Z., Chakraborti, T., Morse, S., Bennett, G.S., and Shaw, G. Four Casein Kinase I isoforms Are Differentially Partitioned between the Nucleus and Cytoplasm. *Exper. Cell Res.* **269,** 275-286 (2001).

28. Sakaguchi, K., Saito, S., Higashimoto, Y., Roy, S., Anderson, C.W., and Appella, E. Damage-mediated Phosphorylation of Human p53 Threonine 18 through a Cascade Mediated by a Casein I-like Kinase. *J. Biol. Chem.* **275,** 9278-9283 (2000).

29. Knippschild, U., Milne, D.M., Campbell, L.E., DeMaggio, A.J., Christenson, E., Hoekstra, M.F., and Meek, D.W. p53 is phosphorylated *in vitro* and *in vivo* by the delta and epsilon isoforms of casein kinase I and enhances the level of casein linase I delta in response to topoisomerase-directed drugs. *Oncogene.* **15,** 1727-1736 (1997).

30. Ho, Y., Mason, S., Kobayashi, R., Hoekstra, M., and Andrews, B. Role of the casein kinase I isoform, Hrr25, and the cell cycle-regulatory transcription factor, SBF, in the transcriptional response to DNA damage in *Saccharomyces cerevisiae. Proc. Natl. Acad. Sci.* **94,** 581-586 (1997).

31. McCay, R.M., Peters, J.M., and Graff, J.M. The Casein Kinase I Family: Roles in Morphogenesis. *Develop. Biol.* **235,** 378-387 (2001).

32. McCay, R.M., Peters, J.M., and Graff, J.M. The Casein Kinase I Family in Wnt Signaling. *Develop. Biol.* **235,** 388-396 (2001).

33. Nishikura, K. A Short Primer on RNAi: RNA-Directed RNA Polymerase Acts as a Key Catalyst. *Cell.* **107,** 415-418 (2001).

34. Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschi T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature.* **411,** 494-498 (2001).

35. Gao, Z.H., Seeling, J.M., Hill, V., Yochum, A., and Virshup, D.M. Casein Kinase I phosphorylates and destabilizes the β-catenin degradation complex. *Proc. Nat. Acad. Sci.* **99,** 1182-1187 (2002).

36. Chijiwa, T., Hagiwara, M., and Hidaka, H. A Newly Synthesized Selective Casein Kinase I Inhibitor, N-(2-Aminoethyl)-5-chloroisoquinoline-8-sulfonamide, and Affinity Purification of Casein Kinase I from Bovine Testis. *J. Biol. Chem.* **264,** 4924-4927 (1989).

37. Zhai, L., Graves, P.R., Longnecker, K.L., DePaoli-Roach, A.A., and Roach, P.J. Recombinant Rabbit Muscle Casein Kinase Iα is Inhibited by Heparin and Activated by Polylysine. *Biochem. Biophy. Res. Comm.* **189,** 944-949 (1992).

38. Behrend, L., Milne, D.M., Stoter, M., Deppert, W., Campbell, L.E., Meek, D.W., and Knippschild, U. IC161, a specific inhibitor of the protein kinases casein kinase I-delta and -epsilon, triggers the mitotic checkpoint and induces p53-dependent postmitotic effects. *Oncogene.* **19,** 5303-5313 (2000).

39. Hall, F.L., Benya, P.D., Padilla, S.R., Carbonaro-Hall, D., Williams, R., Buckley, S., and Warburton, D. Transforming growth factor-β type-II receptor signaling: intrinsic/associated casein kinase activity, receptor interactions and functional effects of blocking antibodies. *Biochem. J.* **316,** 303-310 (1996).

40. Flotow, H. and Roach, P.J. Role of Acidic Residues as Substrate Determinants for Casein Kinase I. *J. Biol. Chem.* **266,** 3724-3727 (1991).

41. Flotow, H., Graves, P.R., Wang, A., Fiol, C.J., Roeske, R.W., and Roach, P.J. Phosphate Groups as Substrate Determinants for Casein Kinase I Action. *J. Biol. Chem.* **265,** 14264-14269 (1990).

42. Macias-Silva, M., Hoodless, P.A., Tang, S.J. Buchwald, M., and Wrana, J.L. Specific Activation of Smad1 Signaling Pathways by the BMP7 Type I Receptor, ALK2. *J. Biol. Chem.* **273,** 25628-25636 (1998).

43. Chen, F., and Weinberg, R.A. Biochemical evidence for the autophosphorylation and transphosphorylation of transforming growth factor β receptor kinases. *Proc. Natl. Acad. Sci.* **92,** 1565-1569 (1995).

44. Fukuchi, M., Imamura, T., Chiba, T., Ebisawa, T., Kawabata, M., Tanaka, K., and Miyazono, K. Ligand-dependent Degradation of Smad3 by a Ubiquitin Ligase Complex of ROC1 and Associated Proteins. *Mol. Biol. Cell.* **12,** 1431-1443 (2001).

45. Wong, C., Rougier-Chapman, E.M., Frederick, J.P., Datto, M.B., Liberati, N.T., Li, J.M., and Wang, X.F. Smad3-Smad4 and AP-1 Complexes Synergize in Transcriptional Activation of

the c-Jun Promoter by Transforming Growth Factor β. *Mol. Cell. Biol.* **19,** 1821-1830 (1999).

46. Yingling, J.M., Datto, M.B., Wong, C., Frederick, J.P., Liberati, N.T., and Wang, X.F. Tumor Suppressor Smad4 is a Transforming Growth Factor β-Inducible DNA Binding Protein. *Mol. Cell. Biol.* **17,** 7019-7028 (1997).

47. Keeton, M.R., Curriden, S.A., van Zonneveld, A.J., and Loskutoff, D.J. Identification of Regulatory Sequences in the Type I Plasminogen Activator Inhibitor Gene Responsive to Transforming Growth Factor β. *J. Biol. Chem.* **266,** 23048-23052 (1991).

48. Satoh, S., Daigo, Y., Furukawa, Y., Kato, T., Miwa, N., Nishiwaki, T., Kawasoe, T., Ishiguro, H., Fujita, M., Tokino, T., Sasaki, Y., Imaoka, S., Murata, M., Shimano, T., Yamaoka, Y., and Nakamura, Y. AXIN1 mutations in hepatocellular carcinomas, and growth suppression in cancer cells by virus-mediated transfer of Axin1. *Nature Genetics.* **24,** 245-250 (2000).

49. Hu, P.C.P., Shen, X., Huang, D., Liu, Y., Counter, C., and Wang, X.F. The MEK Pathway is Required for Stimulation of $p21^{WAF1/CIP1}$ by Transforming Growth Factor-β. *J. Biol. Chem.* **274,** 35381-35387 (1999).

50. Liberati, N.T., Datto, M.B., Frederick, J.P., Shen, X., Wong, C., Rougier-Chapman, E.M., and Wang, X.F. Smads bind directly to the Jun family AP-1 transcriptional factors. *Proc. Natl. Acad. Sci.* **96,** 4844-4849 (1999).

51. Kikuchi, A. Regulation of β-catenin Signaling in the Wnt Pathway. *Biochem. Biophy. Res. Comm.* **268,** 243-248 (2000).

52. Smalley, M.J. and Dale, T.C. Wnt signaling in mammalian development and cancer. *Cancer and Metastasis Review.* **18,** 215-230 (1999).

53. Waltzer, L. and Bienz, M. The control of β-catenin and TCF during embryonic development and cancer. *Cancer and Metastasis Review.* **18,** 231-246 (1999).

54. Hata, A.. TGFβ Signaling and Cancer *Exper. Cell Res.* **264,** 111-116 (2001).

55. Massague, J. and Chen, Y.G. Controlling TGF-β signaling. *Gene. Develop.* **14,** 627-644 (2000).

56. Massague, J. and Wotton, D. Transcriptional control by the TGF-β/Smad signaling system. *EMBO J.* **19,** 1745-1754 (2000).

57. Nishita, M., Hashimoto, M.K., Ogata, S., Laurent. M.N., Ueno, N., Shibuya, H., and Cho, K.W.Y. Interaction between Wnt and TGF-β signaling pathways during formation of Spemann's organizer. *Nature.* **403,** 781-785 (2000).

Appendices

Figure Legends

Figure 1: CKIε interacts with receptor activated smads *in vitro.* Radiolabled CKIε was incubated with GST purifed smads bound to glutathione conjugated to sepharose beads, washed with binding buffer, run on a SDS-PAGE gel, dried and exposed to film to visualize.

Figure 2: CKIγ2-ΔN interacts with smads *in vitro.* Radiolabled smad proteins were incubated with GST-purifed CKIγ2-ΔN bound to glutathione conjugated sepharose beads, washed with binding buffer, run on a SDS-PAGE gel, dried and exposed to film to visualize.

Figure 3: CKIε interacts with TGF-β/BMP receptors *in vitro.* Radiolabled CKIε was incubated with GST purifed TGF-β type I and type II receptors and BMP type I receptor bound to glutathione conjugated to sepharose beads, washed with binding buffer, run on a SDS-PAGE gel, dried and exposed to film to visualize.

Figure 4: CKIε binds to smads and TGF-β type I and type II receptors *in vivo.* Wild type HaCaT (-) cells and HaCaT cells stably expressing CKIε (C) were used to make whole cell lysates. The lysates were then incubated with antibodies to TGF-β type I receptor (TRI), TGF-β type II receptor (TRII), smads2/3 (S2/3), and smads1/5 (S1/5). The antibodies were then precipitated using a 50/50 mixture of ProteinG and ProteinA conjugated to sepharose beads. The immunoprecipitated complexes were then washed, run on a SDS-PAGE gel transferred to PVDF membrane and blotted for CKIε.

Figure 5: CKIε interaction with Smad2/3 *in vivo* is transiently disrupted by TGF-β treatment. HaCaT cells stably expressing CKIε were used to make whole cell lysates. The lysates were then incubated with an antibody to smads2/3. The antibody was then precipitated using a 50/50 mixture of ProteinG and ProteinA conjugated to sepharose beads. The immunoprecipitated complexes were then washed, run on a SDS-PAGE gel transferred to PVDF membrane and blotted for CKIε.

Figure 6: CKIε interaction with TGF-β type II receptor *in vivo* is independent of TGF-β treatment. HaCaT cells stably expressing CKIε were used to make whole cell lysates. The lysates were then incubated with an antibody to the TGF-β type II receptor (TBRII). The antibody was then precipitated using a 50/50 mixture of ProteinG and ProteinA conjugated to sepharose beads. The immunoprecipitated complexes were then washed, run on a SDS-PAGE gel transferred to PVDF membrane and blotted for CKIε.

Figure 7: CKIε acts to fine tune SBE-Lux responsiveness to TGF-β. HepG2 cell were transiently transfected with the reporter construct SBE-Lux, and increasing concentrations of either wild type CKIε or the kinase dead version (KD). Cells were treated with TGF-β overnight following transfection and then harvested and assyed for luciferase activity. Transfection efficiency was corrected using β-galactosidase as an internal control.

Figure 8: CKIε enhancement of 3TP-Lux responsiveness to TGF-β requires smad3 but not smad2. HepG2 cell were transiently transfected with the reporter construct 3TP-Lux, and CKIε alone, smad3 alone, smad2 alone, smad3 and CKIε together, or smad2 and CKIε together. Cells were treated with TGF-β overnight following transfection and then harvested and assyed for luciferase activity. Transfection efficiency was corrected using β-galactosidase as an internal control.

Figure 9: CKIε enhances smad3 activation of the SBE-Lux reporter. HepG2 cell were transiently transfected with the reporter construct SBE-Lux, and CKIε alone, smad3 alone, or smad3 and CKIε together. Cells were treated with TGF-β overnight following transfection and then harvested and assyed for luciferase activity. Transfection efficiency was corrected using β-galactosidase as an internal control.

Figure 10: CKIε phosphorylates smads and TGF-β type II receptor *in vitro*. Smad proteins and the cytoplasmic domains of the TGF-β type I and type II receptors were fused to GST, purified using glutathione conjugated sepharose beads and eluted from the beads using free glutathione. These purified proteins were then incubated with purified CKIε in the presence of ATP-P^{32} for 30 minutes. The reactions were terminated and run on a SDS-PAGE gel, dried and exposed to film to visualize.

Figure 1: CKIε Interacts with Smads In Vitro

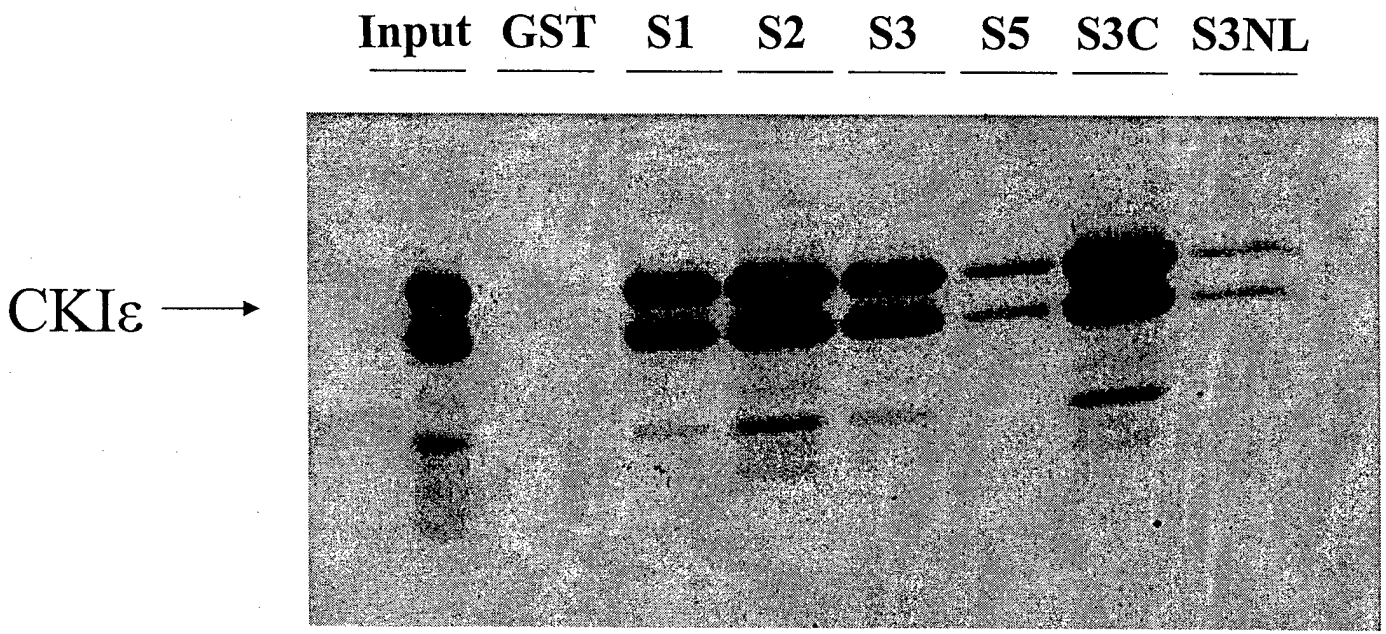


Figure 2: CKIγ2-ΔN Interacts With Smads *in vitro*

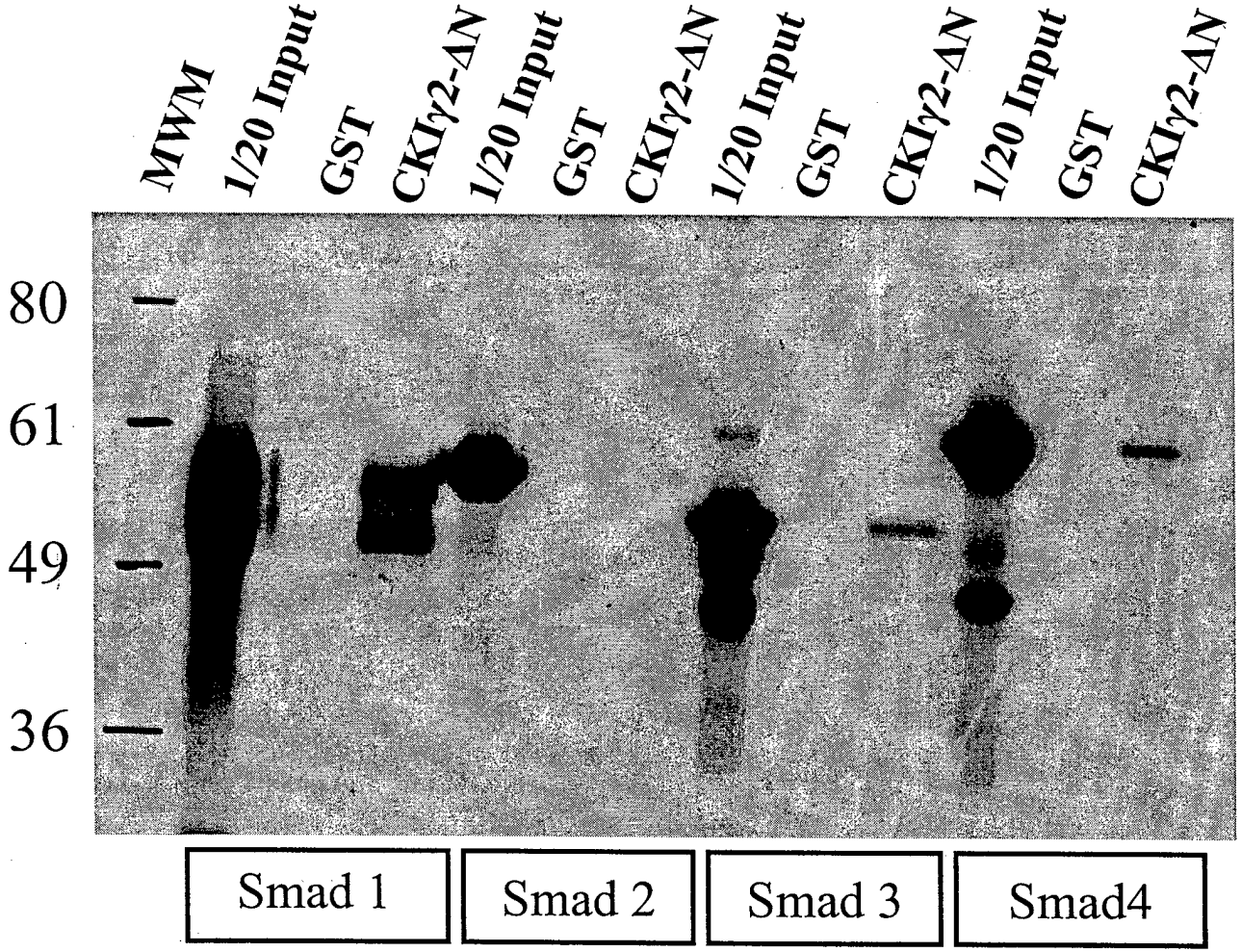


Figure 3: CKIε Interacts with TGF-β/BMP Receptors In Vitro

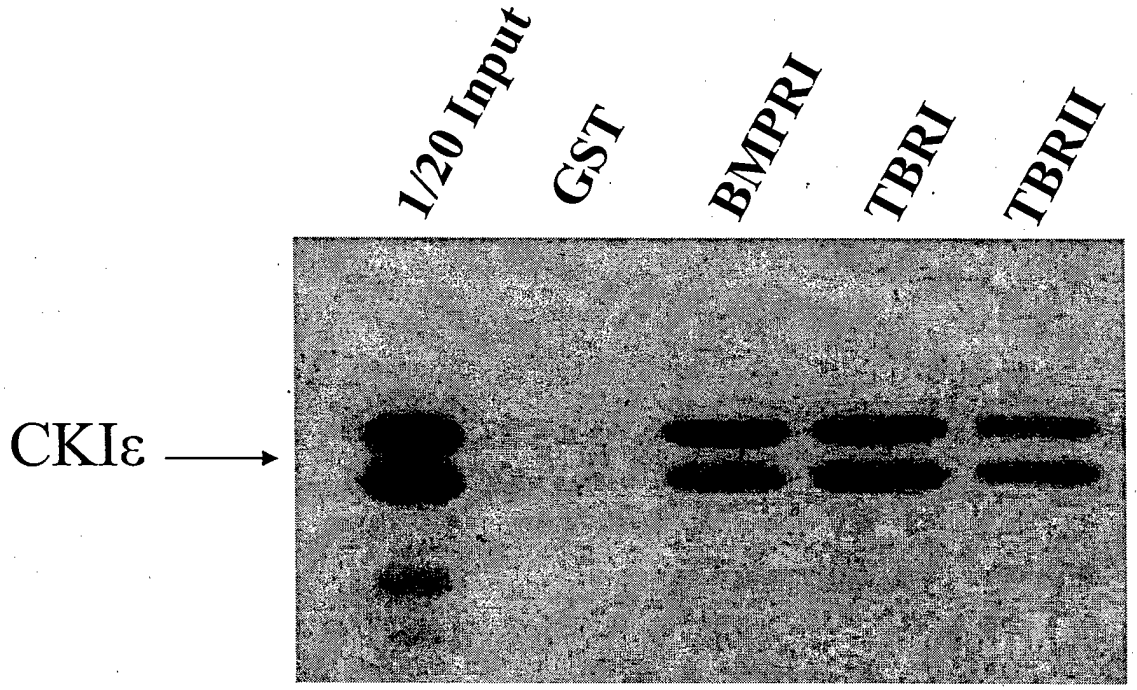


Figure 4: CKIε Binds to Smads and TGF-β Type I and Type II Receptors In Vivo

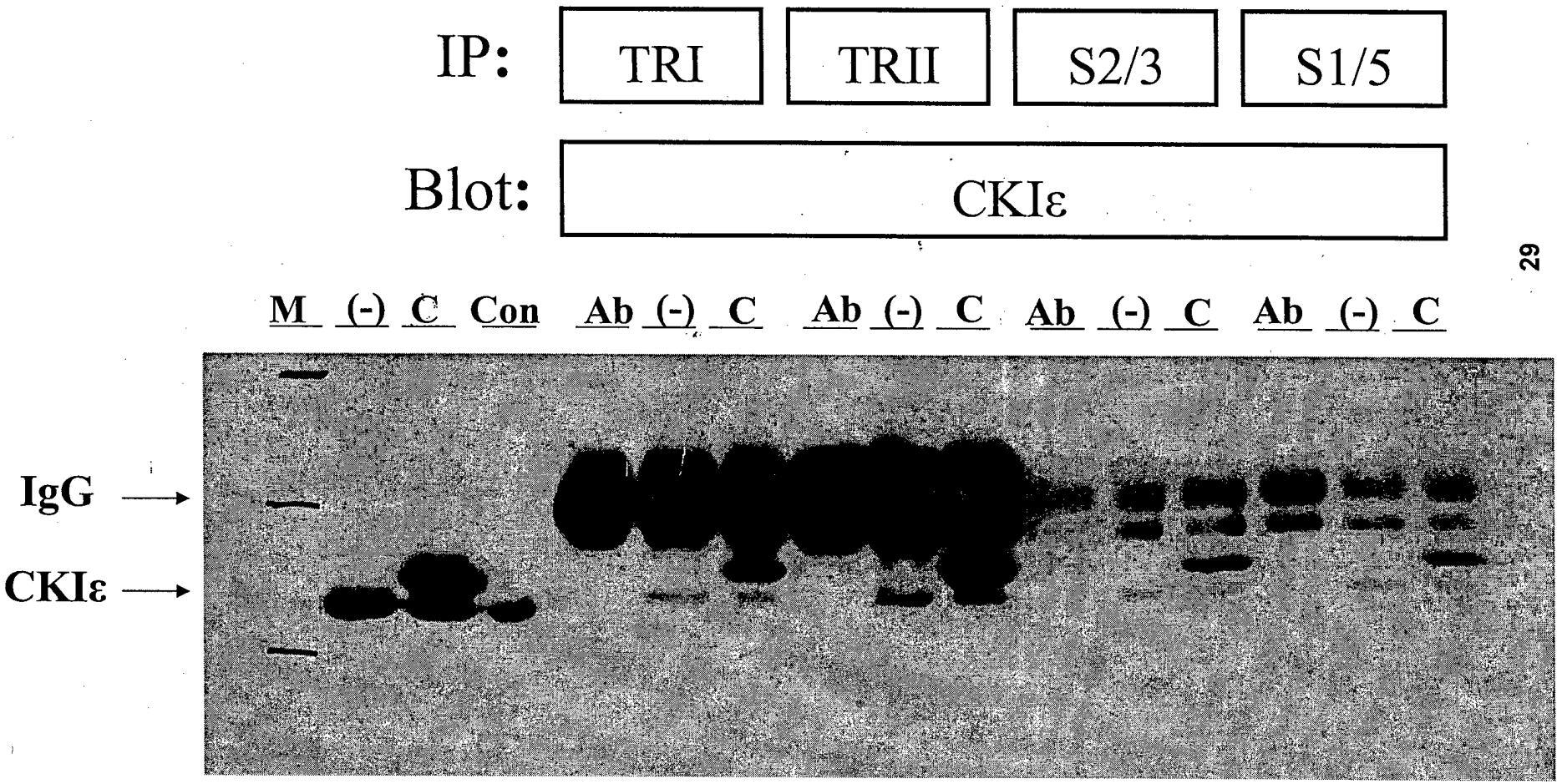


Figure 5: CKIε Interacts with Smad2/3 *in vivo*

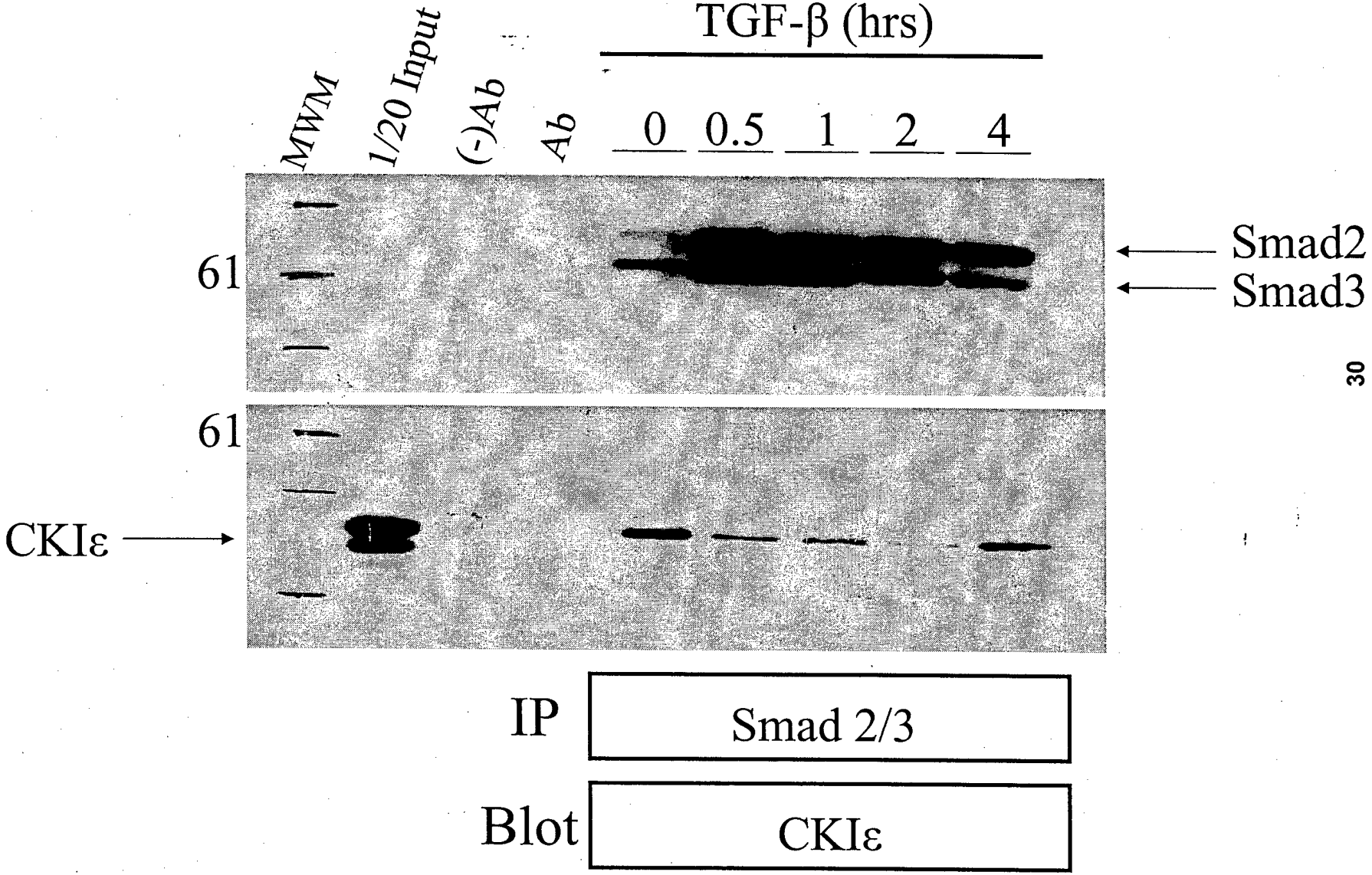


Figure 6: CKIε Interacts with TGF-β Type II Receptor *in vivo*

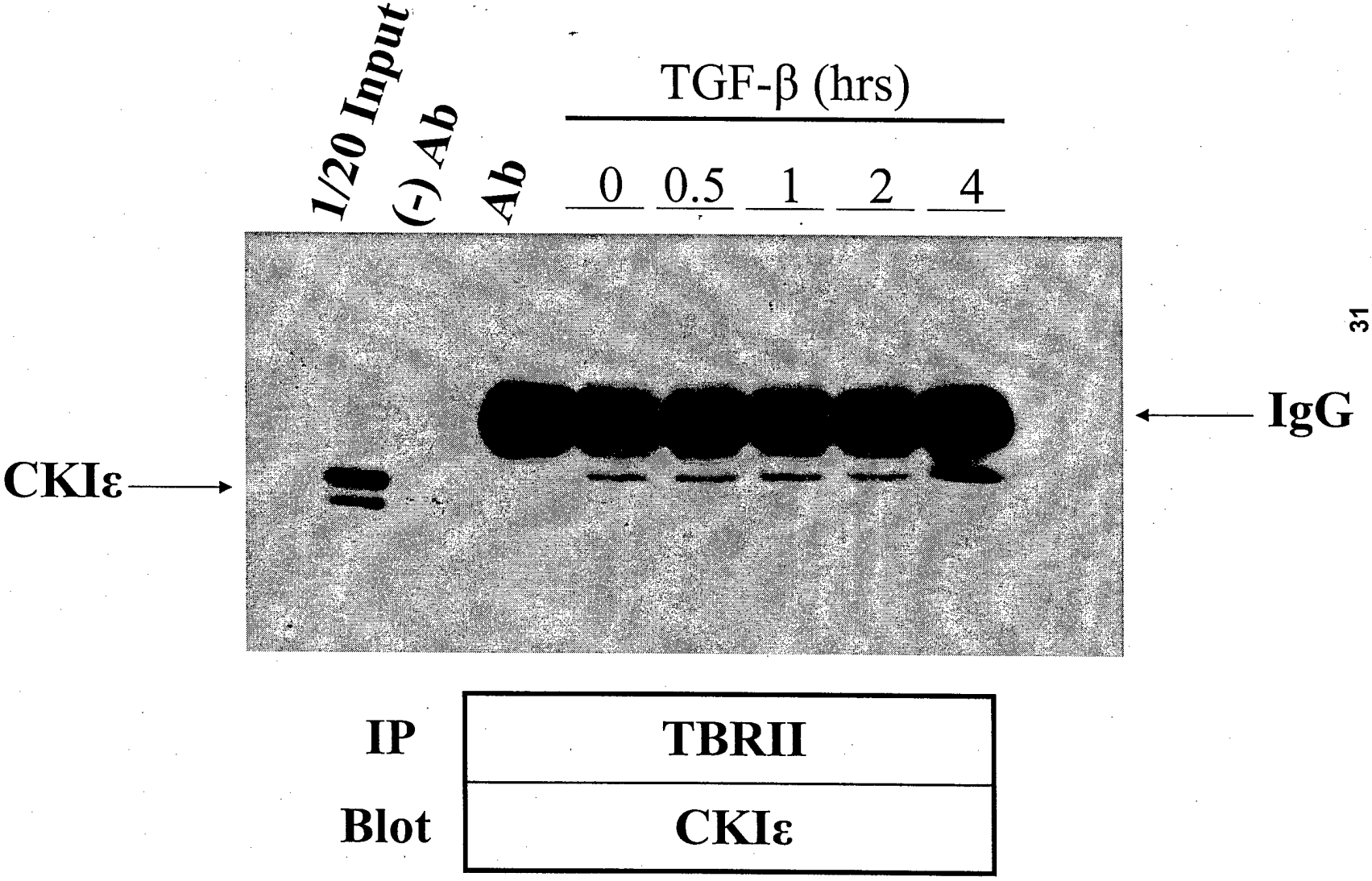


Figure 7: CKIε Acts To Fine Tune SBE-Lux Responsiveness to TGF-β

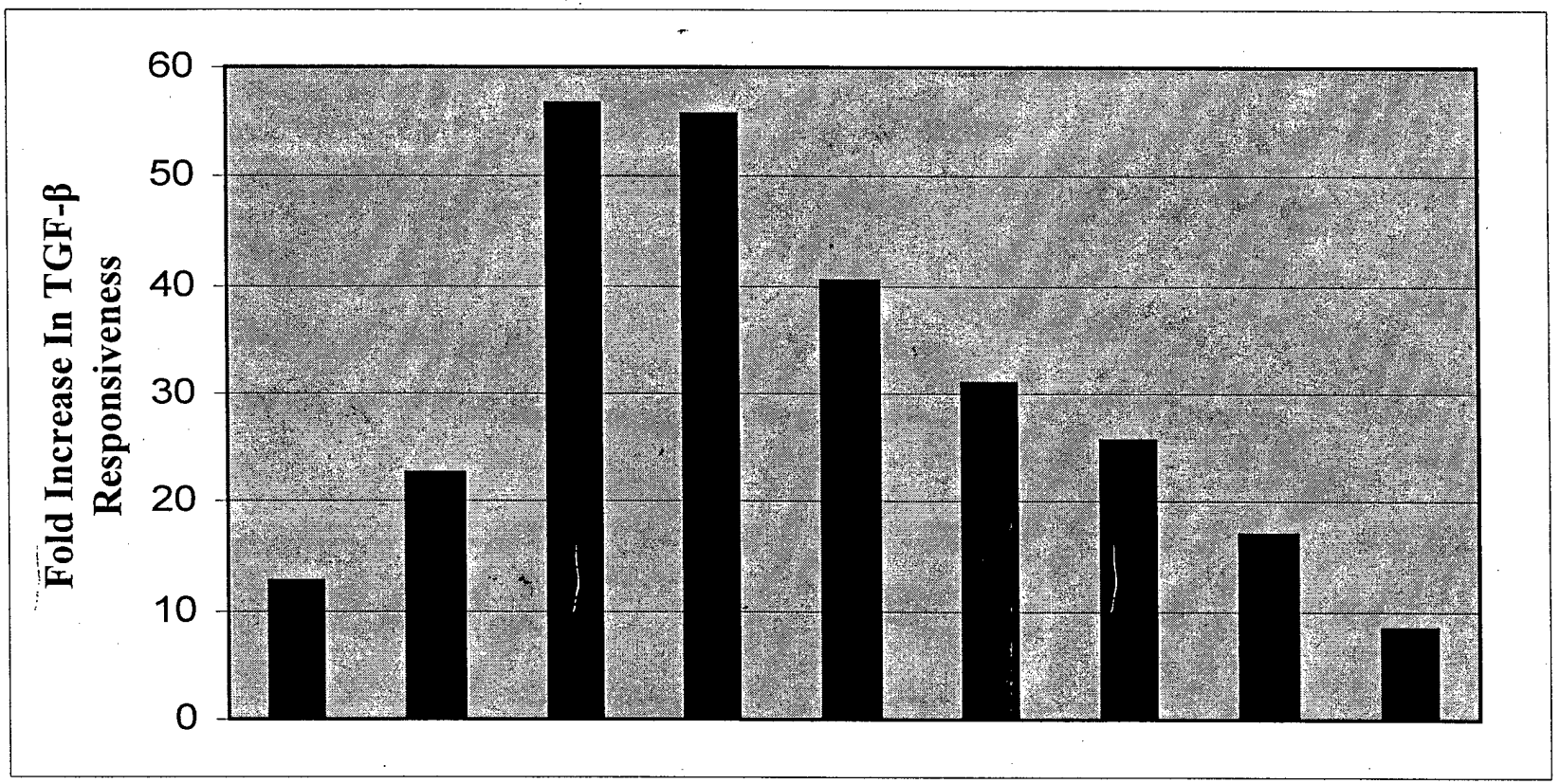
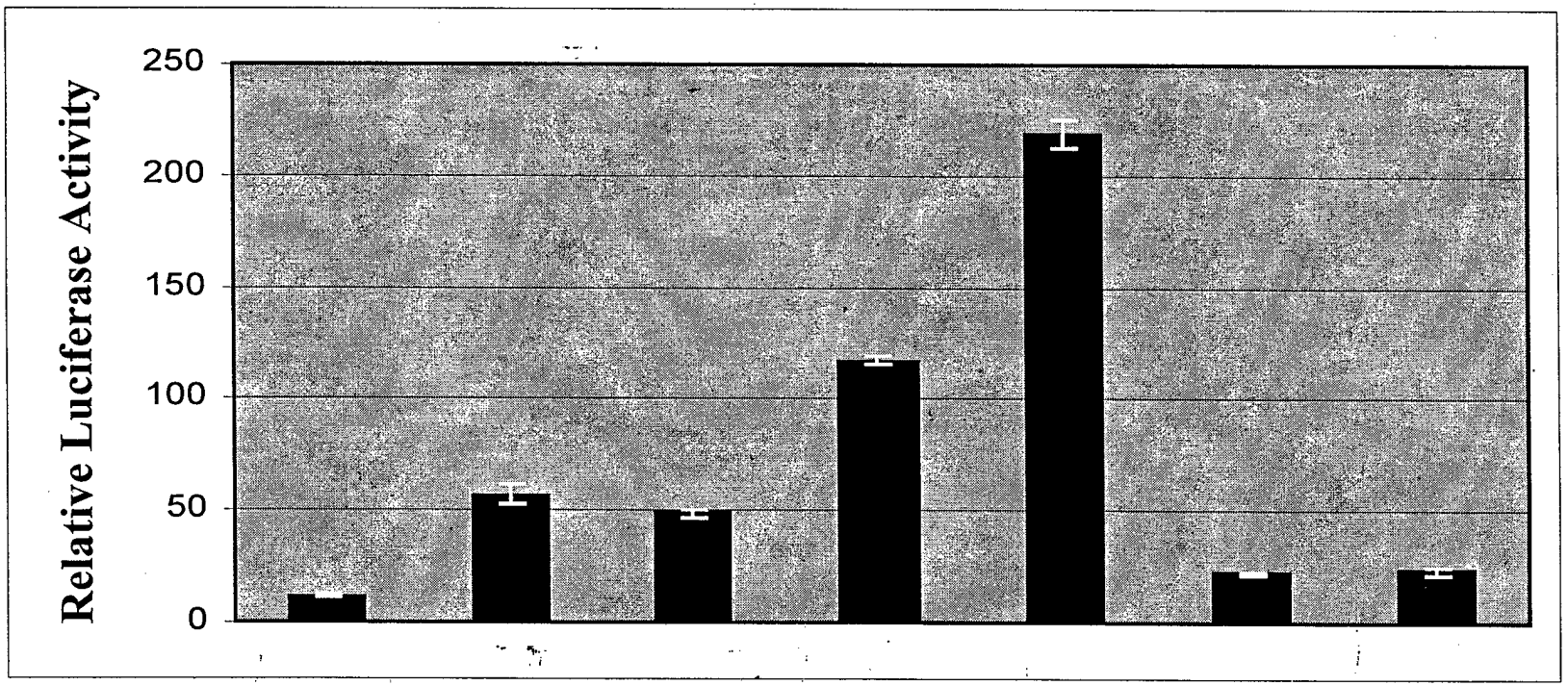
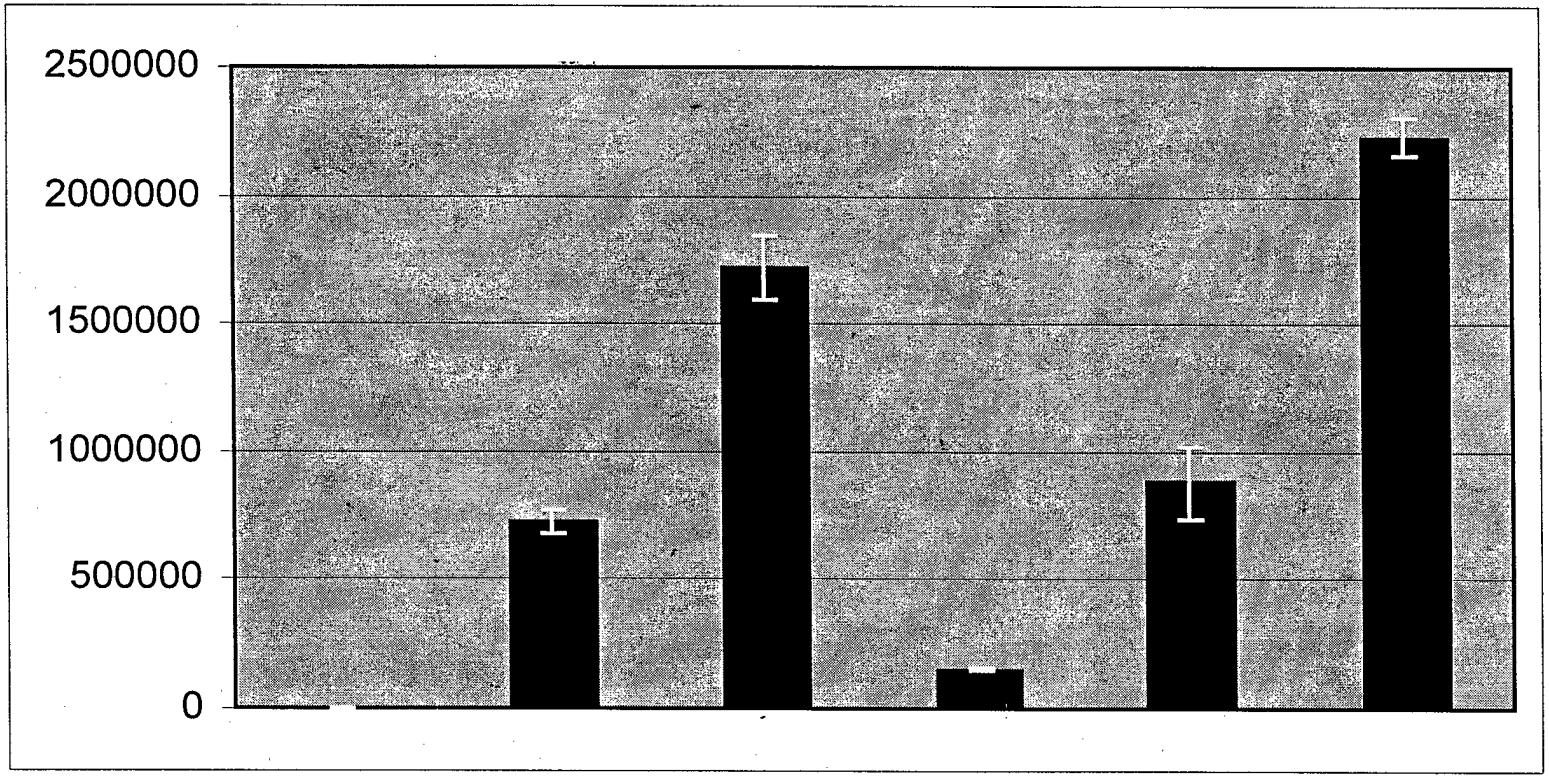


Figure 8: CKIε Enhancement of 3TP-Lux Responsiveness to TGF-β Requires Smad3 but not Smad2



CKIε	-	+	-	+	-	+
Smad3	-		+		-	
Smad2	-				+	
TGF-β	-	+				

Figure 9: CKIe Enhances Smad3 Activation of the SBE-Lux Reporter



Smad3	-	+	-	+
CKIε	-	+	-	+
TGF-β	-		+	

Figure 10: CKIε Phosphorylates Smads and TGF-β Type II Receptor In Vitro